Next Generation Sequencing for microRNA profiling in the porcine endometrium

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The maternal recognition of pregnancy and embryo implantation are the key events occurring during early stages of pregnancy. Sequential changes in expression of numerous genes including growth factors, cytokines and lipid mediators, released locally by endometrium or embryos are crucial for establishment of specific embryo-maternal dialogue (Cha *et al*. 2012). Asynchrony between developing embryos and the uterus may have a tremendous effect on pregnancy, disturbing the implantation and placenta growth, ultimately causing spontaneous abortions as shown in rodents and domestic animals (Song *et al*. 2002, reviewed in Geisert and Yelich 1997). At the physiological state plethora of strictly coordinated embryo-maternal interactions affect gene expression that can be manifested by differences observed at the level of transcriptome and proteome (Cha *et al*. 2012). Interestingly, gene expression may be regulated through posttranscriptional gene regulation mechanisms driven by microRNAs (miRNAs). These small (~22 nucleotides) non-coding RNAs bind to target transcripts leading to inhibition of protein synthesis and/or degradation of the mRNA. Although, miRNAs have been shown to regulate gene expression during implantation in rodents (Hu *et al*. 2008) and are associated with embryo implantation defects in humans (Revel *et al*. 2011), expression profiles and function of miRNAs in the female reproductive tract of domestic animals are poorly identified.

Our previous study has demonstrated differential expression of miRNAs in the porcine embryos/trophoblasts collected between days 10 and 20 of pregnancy (Krawczynski *et al*. 2012), suggesting possible involvement of miRNAs in embryo development and implantation. In the present study we have applied Next Generation Sequencing (NGS) technology to analyze miRNA expression in porcine endometrial samples. Gilts in third estrus were either inseminated or let to undergo the next estrous cycle. Samples were collected on days 12, 16 and 20 of either the estrous cycle or pregnancy (n = 6/day/status). Total RNA containing small RNA fraction was extracted by the mirVana miRNA isolation kit and used for sequencing. Small RNA libraries were constructed using the NEXTflex™ Small RNA Sequencing Kit. Briefly, 100 ng of total RNA containing miRNA was ligated with the 3′ and 5′ adaptors. Then, the reverse transcription mix was added to the adaptor-ligated RNA to synthesize the cDNA. In the PCR amplification step specific barcodes for multiplexing were added. Resulting PCR products were pooled and size fractioned on an agarose gel to obtain ~140 bp fragments, which were sequenced using TrueSeq SR Cluster Kit v. 2 and Illumina Genome Analyzer IIx.

Generally, 36 libraries generated 22,391 to 69,065 unique sequences, which demonstrated predominant length of 22 nt and GC content at the level of ~40%. Abovementioned sequences were further blasted against miRBase v.18, Ensembl and NCBI databases. As a result, 815 sequences were annotated to known/predicted porcine and mammalian, mature or stem-loop miRNA sequences. 115 sequences showed 100% homology to known porcine mature miRNAs, 518 sequences showed full homology to miRNA stem-loop structures present in the porcine genome, 68 and 103 sequences were annotated to known mammalian, mature and stem-

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loop miRNAs, respectively. Finally, 8 and 3 sequences were found to have 100% homology to predicted stem-loop structures deposited in Ensembl and RefSeq database, respectively. Different length and sequence variants (IsomiRs) for vast majority of canonical miRNAs were observed, which is common in NGS studies of small RNA libraries (reviewed by Neilsen *et al*. 2012). Most of miRNAs demonstrated modifications at the 3´-terminus represented as nucleotide addition and/or trimming with the most common single base addition of uracil (42%) and the least frequent guanidine (17.5%). However, the 5´-terminus of some miRNAs was also affected. The BioConductor package Limma was used to find differentially expressed canonical miRNAs. This analysis revealed 70 differentially expressed miRNAs between pregnant and cyclic gilts ($p \leq 0.05$). Ingenuity® Pathway analysis software was used to find the biological processes and pathways associated with differentially expressed canonical miRNAs (Fisher's exact test, $p < 0.05$). The mostly affected molecular and cellular functions during pregnancy were identified: a) cell cycle (2-5 miRNAs), b) cellular development (3-6 miRNAs), c) growth and proliferation (3-5 miRNAs). Computational target prediction analysis using miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) revealed that some up- and down-regulated canonical miRNAs, e.g. miR-26a-5p, miR-181a-5p or miR-205 may regulate expression of estrogen receptor α, leukemia inhibitor factor and its receptor, vascular endothelial growth factor, fibroblast growth factor 2 and 7 or Wnt5a; genes known to be involved in establishment of embryo-maternal interaction during early stages of pregnancy in the pig.

In conclusion, the present results showed the expression of miRNAs and isomiRs in the porcine endometrium during the estrous cycle and early pregnancy in pigs. Identified canonical miRNAs were predicted to regulate expression of target genes crucial for cyclic changes of endometrium during the estrous cycle or these observed when pregnancy occurs. Since, miRNAs can be transferred between cells to facilitate cell-to-cell interactions (Valadi *et al*. 2007), together with our previous results, it seems likely that miRNAs of embryo and uterine origin are substantial elements of embryo-maternal crosstalk during early pregnancy in the pig.

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